Arachidonic Acid Stimulates DNA Synthesis in Brown Preadipocytes through the activation of Protein Kinase C and MAPK

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Abstract

Arachidonic acid (AA) is a polyunsaturated fatty acid that stimulates the proliferation of many cellular types. We studied the mitogenic potential of AA in rat brown preadipocytes in culture and the signaling pathways involved. AA is a potent mitogen which induces 4-fold DNA synthesis in brown preadipocytes. The AA mitogenic effect increases by NE addition. AA also increases the mitogenic action of different growth factor combinations. Other unsaturated and saturated fatty acids do not stimulate DNA synthesis to the same extent as AA. We analyzed the role of PKC and MEK/MAPK signaling pathways. PKC inhibition by bisindolilmaleimide I (BIS) abolishes AA and phorbol ester stimulation of DNA synthesis and reduces the mitogenic activity of different growth factors in brown preadipocytes. Brown preadipocytes in culture express PKC α, δ, ε and ζ isoforms. Pretreatment with high doses of the phorbol ester PDBu, induces downregulation of PKC ε and δ and reproduces the effect of BIS indicating that AA-dependent induction of DNA synthesis requires PKC activity. AA also activates MEK/MAPK pathway and the inhibition of MEK activity inhibits AA stimulation of DNA synthesis and brown adipocyte proliferation. Inhibition of PKC-δ by rottlerin abolishes AA-dependent stimulation of DNA synthesis and MAPK activation, whereas PKC-ε inhibition does not produce any effect. In conclusion, our results identify AA as a potent mitogen for brown adipocytes and demonstrate the involvement of the PDBu-sensitive PKC-δ isoform and MEK/MAPK pathway in AA-induced proliferation of brown adipocytes. Increased proliferative activity might increase the thermogenic capacity of brown fat.

Introduction

Arachidonic acid (AA; 20:4 n-6; 5,8,11,14-eicosatetraenoic acid) is a polyunsaturated ω-6 essential fatty acid (PUFA), essential constituent of the diet that is available to the cells from the extracellular environment or through the release from its esterified form in membrane phospholipids and triglycerides. The intake of AA is about 0.2 g/day, but AA is also produced from linoleic acid which daily intake is much higher. In general, triglycerides are less rich in AA than phospholipids. Only in those tissues where the amount of neutral lipids exceeds the amount of phospholipids, such as adipose tissue, neutral lipids can contribute significantly to the free AA concentration. AA can be released from its esterified form in membrane phospholipids by the action of the phospholipase A2 (PLA2) [1]. It can also be generated from diacylglycerol (DAG), released from phospholipids by the action of phospholipase C (PLC) or phospholipase D; the enzymes diacylglycerol lipase or monoacylglycerol lipase can produce AA from DAG. AA is then metabolized into compounds of a greater biological activity such as prostaglandins and thromboxanes via the cycloxygenase system, and to leukotrienes and hydroxyeicosatetraenoic acids (HETEs) via the lipoxygenase pathway [2].

AA has a role in many biological processes, such as chemotaxis, inflammation and signal transduction [3, 4]. In addition to these functions, AA has a role in the stimulation of cell proliferation. The release of AA from the membrane phospholipids constitutes one of the signals involved in the stimulation of cellular proliferation. Growth factors such as EGF, PDGF, FGF and bombesin stimulate the releasing of AA in many cellular types [5-9] and it has been shown that the induction of mitogenesis by these growth factors requires AA metabolism.

In many cases, the stimulation of cell proliferation in response to AA is mediated by the metabolites of its oxidation. In Swiss 3T3 fibroblasts, stimulation of cellular proliferation by AA is mediated by conversion of AA to prostaglandin E2 (PGE2) and subsequent stimulation of PKC [10, 11]. AA can modulate the activity of different components of the intracellular

signaling [12-14], specially PKC isoforms [15]. Not only AA but also other unsaturated fatty acids directly activate certain PKC isoforms [16] and induce its traslocation [17, 18].

In hamster brown adipocytes AA is released in response to NE, the main regulator of brown adipocyte function, via α1 adrenergic receptors [19]. NE-induced AA release can be produced by the action of PLA2 or by the sequential action of PLC and diacylglycerol lipase. Triglycerides accumulated in the lipid droplets of brown adipocytes constitute another important source of AA in the adipose tissues. It has been described that the lipolytic stimulation elicited by intracellular cAMP increases, promotes the release of AA from the lipid droplets in brown adipocytes [19] .

In view of these antecedents it is clear that AA is an important component of brown adipose tissue that can be released in response to the principal regulator of brown adipocyte function, norepinephrine (NE), but no data has been reported about its physiological function. Herein we described the mitogenic effect of AA on brown preadipocytes and the importance of PKC for its mitogenic action. This fact has implications on the increase in BAT mass produced during adrenergic stimulation.

Materials and Methods

Materials

Dulbecco Modified Eagle's medium (DMEM) was obtained from Gibco (Uxbridge, U.K.). Newborn calf serum (NCS) was obtained from Flow (Paisley, Scotland). Antibiotics were obtained from a local pharmacy. Bovine serum albumin (BSA in solution at 22%, pH=7.2) was purchased from Ortho Diagnostic Systems, Johnson & Johnson Co. (Raritan, NJ). Collagenase, bovine insulin, ascorbic acid, norepinephrine (NE), vasopressin, nordihydroguaiaretic acid (NDGA), indomethacin, phorbol 12, 13-dibutyrate (PDBu), AA, palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LA) and linolenic acid (LnA) were purchased from Sigma (St. Louis, MO). Acidic and basic fibroblast growth factor (aFGF and bFGF), platelet-derived growth factor (PDGF) (B/B homodimer) and epidermal

growth factor (EGF) were from Boehringer Mannheim (Mannheim, Germany). aFGF was used in the presence of 50 μg/ml heparin (final concentration), as recommended for proper biological activity. Bisindolylmaleimide-I (BIS) and PD98059 were obtained from Calbiochem (Darmstadt, Germany). Specific antibodies for the different PKC isoforms were a generous gift from Dr. Lisardo Bosca. Rabbit polyclonal ERK sc-94, mouse monoclonal pERK sc-7383, goat anti-mouse IgG-HRP sc-2005 and goat anti-rabbit IgG-HRP sc-2004 were from Santa Cruz Biotechnology, Inc. Rottlerin was used as PKCδ inhibitor and the peptide Myr-SIYRRGARRWRKL-OH as PKCζ substrate inhibitor (both from Calbiochem). The octapeptide N-Myristoyl-EAVSLKPT (Isogen) was used as PKCε translocation inhibitor. Streptavidin-horseradish peroxidase conjugate secondary antibodies, the chemiluminescence kit and ^{3}H-thymidine were from Amersham Intern. (Buckinghamshire, U.K.). PVDF membranes were purchased from Dupont and the glassfiber filtermats for thymidine incorporation were from Wallac Oy (Turku, Finland). All other chemicals were reagent grade or molecular biology grade.

Cell isolation and culture

Brown fat precursor cells were isolated from the interscapular brown adipose tissue (BAT) of 20 day-old rats, as described by Néchad et al. [20]with the difference that we did not perform the hypoosmotic shock. The process involved collagenase digestion, separation of mature adipocytes by flotation and subsequent filtration through 25 μm silk filters, obtaining precursor cells by centrifugation. The precursor cells obtained from each animal were divided into two culture flasks (Nunclon, 25 cm^{2}, Nunc, Roskilde, Denmark), each containing 5 ml of culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.5 nM insulin, 10 mM Hepes, 50 IU penicillin/ml, 50 μg streptomycin/ ml, 25 μM sodium ascorbate (culture medium) and 10% newborn calf serum (NCS). The cells were

incubated at 37 C in an atmosphere of 5% CO_2 in air with 95% humidity. Cells were washed on day 1 (2000 cells/cm^2) and culture medium was changed every other day.

For proliferation assays, preconfluent cells (day 2-3) were harvested using 0.2% collagenase in DMEM, washed and seeded in 24 multiwell tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at a density of 8000 cells/ cm^2, using 1 ml of culture medium supplemented with 10% NCS. After 12 h the cells were washed twice with medium and maintained for 48 hours in culture medium supplemented with 0.2% BSA (time 0, quiescient cells). This was the starting point for mitogenic stimulation.

Proliferation assays

Growth factors, hormones or serum were added to the cells at time 0 [21], at the concentrations indicated in each experiment. For ^{3}H-thymidine incorporation assays, quiescent cells were stimulated with the appropriate mitogens and hormones at time 0, in the presence of ^{3}H-thymidine (1 μCi/ml). After 40 hours of exposure, the medium was discarded and the cells were removed from the plate, using a trypsin-EDTA solution. Thereafter, the contents of each well were harvested onto glass-fiber filters using a cell harvester from Innotech AG (Dottikon, Switzerland) and the radioactivity incorporated into DNA was determined using a beta-scintillation counter (MicroBeta) from Wallac Oy (Turku, Finland). The conditions used for the proliferation assays were validated in our previous studies [21].

Protein extracts and Western blotting

Primary cultures of brown fat cells in day 3 were maintained for 48 hours in culture medium supplemented with 0.2 % BSA to induce quiescence. For downregulation experiments, cells were treated with 0.2 or 2 μM PDBu during the last 24 hours of the quiescence induction period. Cells were then treated with different inhibitors, growth factors, hormones or fatty acids at the concentrations and times indicated for each experiment. Afterwards, cells were rinsed with PBS and harvested in a solution containing 20 mM Tris,

pH=8, 5 mM EGTA, 1% Triton X-100 and 10 μg/ml leupeptin (lysis buffer). Cellular homogenates were centrifuged in a microcentrifuge for 10 min at 4ºC to clarify the extracts. The amount of proteins was determined by the method of Lowry [22]. Protein extracts were heated at 90ºC 10 min in the presence of Laemmli buffer and then kept at –70ºC. Thirty micrograms of total protein extracts were subjected to 10% PAGE and transferred to PVDF (Dupont) membranes with a transfer buffer containing 190 mM glycine, 25 mM Tris, 0.033% SDS and 20% methanol. Membranes were blocked with TTBS buffer (20 mM Tris, 137 mM ClNa pH 7.6 and 0.1 % Tween 20) and 5% skimmed milk, and incubated with the primary antibody diluted 1:3000 in TTBS, for 2 hours at 4ºC. After washing in TTBS buffer, membranes were incubated with the secondary antibody diluted 1:1000 in TTBS and 5 % non-fat dried milk, for 2 hours at 4ºC. The visualization of the bands was performed with the Enhanced Chemiluminescence (ECL) kit (Amersham) and autoradiographed. Membranes were stripped at 50ºC with a solution containing 62.5 mM Tris HCl pH 7.5, 2 % SDS and 100 mM beta-mercaptoethanol.

Statistic analysis

Mean values ± SD given were obtained from at least 2 different culture flasks or wells. All experiments presented were repeated at least 3 times and representative experiments are shown. Data were submitted to one-way ANOVA, after testing for homogeneity of variance using Bartlett's procedure for groups of unequal size. All statistical calculations were performed as following the method described by Snedecor and Cochran [23].

Results

AA-dependent stimulation of DNA synthesis in brown adipocytes.

AA (20:4n-6) is a poly-unsaturated fatty acid that has been reported to stimulate the proliferation of many cellular types. In this study we wanted to test the mitogenic activity of AA in brown adipocytes in culture. For this purpose, quiescent brown adipocytes were treated

with increasing concentrations of AA (1, 10 and 50 μM) and thymidine incorporation was measured 40 hours later. The results represented in Figure 1A show that addition of 1 μM AA produces a 2-fold increase in thymidine incorporation, relative to untreated cells. Maximal increases are achieved at 10 μM AA (4.8 fold increase), an effect that persists at 50 μM. Addition of NE (1 μM), the main hormone regulating BAT function, increases AA stimulation of DNA synthesis (Figure 1B).

One of our interests relative to BAT function is to identify growth factors and hormones that stimulate DNA synthesis and cell division in brown preadipocytes to define mitogenic conditions for brown preadipocytes. In that sense in a previous work we defined some mitogenic combinations that included polypeptide growth factors (EGF, PDGF, aFGF or bFGF), vasopressin and NE [21]. Here we show that addition of AA to these mitogenic combinations increases thymidine incorporation in most of the conditions, reaching levels comparable to that obtained in response to 10% serum (NCS) (Figure 1C).

To test if the mitogenic action of AA was due to its metabolites, we treated quiescent brown adipocytes with 1, 10 and 50 μM AA in the presence of NDGA (inhibitor of the lipoxygenase and epoxygenase pathways) or indomethacin (inhibitor of the cycloxygenase pathway) and none of them had a significant effect on DNA synthesis (Figure 1D). The doses tested were 0.1 μM to 20 μM for indomethacin and 0.05 μM to 5 μM for NDGA (not shown). These results indicate that the metabolites of AA are not essential for the effect of AA on DNA synthesis.

The mitogenic activity of AA was compared to that of other unsaturated fatty acids including OA, LA (the precursor of AA) and LnA. The effect of the saturated fatty acids PA and SA was also analyzed. Quiescent brown preadipocytes were treated with 10 μM or 50 μM of the different fatty acids and thymidine incorporation was measured 40 hours later. The results represented in Figure 1E show that DNA synthesis stimulation in response to the unsaturated fatty acids LA, LnA and OA is slightly higher than that observed in response to the saturated acids SA and PA, but much lower than the AA effect. It can be concluded that AA

behaves as a potent mitogen for brown preadipocytes and that its effect on DNA synthesis stimulation is stronger than that elicited by other fatty acids.

Role of PKC on the stimulation of DNA synthesis by AA

PKC has been implicated in the transduction of signaling pathways leading to cellular proliferation in response to many different growth factors, hormones and also AA. Our next objective was to analyze the involvement of PKC on DNA synthesis stimulation elicited by AA. To this end, we first determined thymidine incorporation in cells treated with AA (10 μM) in the presence of the PKC inhibitor, BIS that inhibits conventional (α, β, γ), novel (ε, δ) and atypical PKC (ζ). It is well known that PKC is the main target of the tumor promoting phorbol esters. The phorbol esters TPA and PDBu stimulate conventional and novel PKCs but not PKCζ. As can be seen in Figure 2A, the presence of BIS (1 μM) abolishes AA-mediated stimulation of brown preadipocytes DNA synthesis and it also inhibits the stimulation of DNA synthesis elicited by TPA (200 nM) and PDBu (40 nM) (Figure 2B).

We also analyzed the contribution of PKC to the mitogenic activity of the growth factors and hormones that we previously identified as mitogens for brown preadipocytes [21] [21]. Figure 2C shows that BIS treatment results in a drastic reduction of thymidine incorporation in response to EGF and vasopressin. BIS also reduces DNA synthesis stimulation by aFGF and bFGF, but to a lesser extent. NE stimulation of DNA synthesis is too low to consider the inhibition by BIS a significant effect.

It is therefore likely that PKC activation is an important event in the stimulation of DNA synthesis in brown preadipocytes since it is required for the mitogenic action of growth factors such as EGF, vasopressin, and for the AA-dependent stimulation of DNA synthesis.

PKC isoforms in brown adipocytes and downregulation by phorbol esters.

It is well known that long treatments with high concentrations of phorbol esters lead to the downregulation of PKC. Phorbol doses and the length of the treatment can affect in a different way the activity of the different isoforms. In general, both conventional and new PKCs are

downregulated by phorbol esters but the atipic PKC subfamily, which include PKC ζ, does not have DAG binding domain and hence these PKCs are not regulated by phorbol esters. Downregulation of PKC induced by phorbol esters has been used extensively in many cellular systems to study the involvement of PKC isoforms in several processes such as cellular proliferation. To study this effect of PKC-downregulation we analyzed by Western blot the expression level of PKC isoforms α, δ, ε and ζ. in quiescent brown preadipocytes, either in untreated cells or treated with 0.2 or 2 μM of the phorbol ester PDBu, for 24 hours. The specificity of the antibodies was checked using brain extracts as positive controls (data not shown). Figure 3A shows that quiescent brown adipocytes express α, δ, ε and ζ isoforms. Immunodetection of γ and β isoforms did not show any specific band (data not shown). The amount of PKC δ and ε became undetectable 24 hours after treatment with 0.2 and 2 μM PDBu, while PKC α and PKC ζ levels remain unaltered, indicating that treatment of brown adipocytes with 0.2 μM PDBu induces downregulation of PKCδ and ε.

We studied the effect of PKC-downregulation on the mitogenic action of PDBu at increasing concentrations (20, 40, 100, 200 and 400 nM). For this purpose, quiescent cells were pretreated for 24 hours with 0.2 μM PDBu prior to the mitogenic stimulation by this phorbol ester. Figure 3B shows the results of this assay. PDBu reaches its maximal effect at 40 nM, decreasing at higher concentrations. In pretreated cells, PKCδ and ε have been downregulated, no DNA synthesis stimulation is observed, although basal thymidine incorporation level is higher than in non-pretreated cells.

When 10 μM AA is added to cells pretreated with 0.2 μM PDBu, and thymidine incorporation is measured, the mitogenic stimulus observed in response to AA decreased substantially, as shown in Figure 3C.

These results confirm the importance of PKC for the mitogenic effect of AA in brown adipocytes and suggest a role for PKCδ and/or PKCε in AA-dependent stimulation of DNA synthesis in brown adipocytes.

Role of MEK activity in the mitogenic action of AA

The signaling pathway involving MEK and MAPK (ERK 1 and 2) activation constitutes a signaling cascade of much relevance for the stimulation of DNA synthesis in many different cellular systems. To analyze the contribution of this pathway to the mitogenic effect of AA, phospho ERK1/2 levels were analyzed by Western blot in response to AA addition to brown adipocytes cultures. The results obtained (Figure 4A) show that 10 μM AA induces ERK1/2 activation, an effect that is inhibited by the specific MEK inhibitor PD98059. The effect of AA is compared to the well-known insulin and PDBu activation of ERK1/2 phosphorylation. Both effects are also abolished by the MEK inhibitor PD98059. We next analyzed the effect of MEK inhibition in the stimulation of DNA synthesis and brown adipocyte proliferation by AA. The results represented in Figure 4B show that the presence PD98059 abolishes AA-dependent stimulation of DNA synthesis and cellular proliferation, indicating that MEK activity is involved in the mitogenic action of AA in brown preadipocytes.

Role of PKCδ in the mitogenic action of AA

It is well known that MEK could be a downstream target of PKC. Previously down-regulation experiments showed the involvement of PKC isoforms δ and ε in the mitogenic effect of AA; now we study whether the inhibition of PKC δ and ε could affect AA-dependent stimulation of ERK1/2 phosphorylation and thymidine incorporation. Pretreatment of brown adipocyte cultures with rottlerin, an inhibitor of PKC δ, prevents ERK1/2 phosphorylation induced by AA (Figure 5A). Specific inhibition of PKCε or PKCζ does not affect pERK1/2 levels obtained by AA treatment, indicating that PKCδ activation is required for AA induction of ERK1/2 phosphorylation. The same results are obtained in response to the phorbol ester

PDBu, i.e., rottlerin (5 μM) prevented PDBu-dependent stimulation of ERK1/2 phosphorylation while PKCε or PKCζ inhibition has no effect. Lower doses of rottlerin (0.5 μM) also prevented ERK1/2 phosphorylation by AA or PDBu though to a lower extent (Figure 5B). Thymidine incorporation studies reveal that the inhibitor of PKCδ, rottlerin, inhibits PDBu and AA-dependent stimulation of DNA synthesis in a dose dependent manner (Figure 5C). PKCε inhibition did not reduce either PDBu or AA effects (Figure 5D). In conclusion, these results indicate that activation of ERK1/2 by PKCδ is required for the stimulation of DNA synthesis by AA in brown preadipocytes.

Discussion

In the present study we investigated the induction of DNA synthesis by AA in brown preadipocytes in primary culture. We found that AA is a potent mitogen for these cells that induces DNA synthesis and cell division. The mitogenic action of AA was enhanced by NE, which acts as a mitogen for brown preadipocytes [21, 24].

In a previous study [21]we demonstrated that proliferation of brown fat cells is regulated by growth factors and hormones and we defined mitogenic combinations that stimulated DNA synthesis in brown adipocytes to an extent similar to the effect produced by 10% serum. Here we show that AA added to these mitogenic mixtures increases thymidine incorporation to a level comparable to the effect of 10% serum [21]. This first evidence underlines the importance of AA as a factor that increases the number of brown adipocytes and potentially its thermogenic capacity.

The mitogenic action of AA might also be exerted by its metabolites produced through cycloxygenase and lipoxygenase pathways. In Swiss 3T3 cells, AA stimulation of cellular proliferation is dependent on PGE2 formation [10], a product of AA oxidation by the action of cycloxygenase activity. This does not seem to be the mechanism operating in brown adipocytes

as the inhibitor of prostaglandin formation, indomethacin, does not block the mitogenic action of AA. The inhibition of the metabolites generated by the lipoxygenase activity by NDGA, did not reduce either the mitogenic effect of AA. Recently AA metabolites, formed by COX2, have shown to induce brown adipocytes within white adipose depots [25, 26] discovering new roles of AA metabolites inducing "browning" in white fat depots.

Here we show that addition of AA to BAT cells stimulates MEK-dependent ERK-1/2 activation. Furthermore, we have stablished that AA-induced ERK-1/2 activation is PKC-dependent, suggesting that PKC is a critical kinase that lies upstream of MEK/ERK in these cells. Using a selective inhibitor of MEK we found that ERK-1/2 inhibition reduced AA induced DNA synthesis. The activation of PKCδ inducing ERK phosphorylation and cell proliferation has been described in other cells [27-29]. These findings indicate that AA addition leads to PKC activation, stimulation of the ERK pathway and induces progression through the cell cycle of these cells arrested in G0/G1. These findings agree with studies using preadipocytes from ERK1-/- mice in primary culture [30] where Pref1 (*dalk1)*, a gene associated to proliferative activity in brown adipocytes [31] show a decreased expression, suggesting that ERK1 is required for a full proliferative activity. ERK null mice have fewer adipocytes and smaller fat pads.

AA has been described as a potent activator of different PKC isoforms [15]. By using the PKC inhibitor BIS we have shown that PKC activity is required for AA activation of DNA synthesis in brown adipocytes. Furthermore, depletion of DAG dependent and calcium independent PKCs, such as PKC δ and ε, by prolonged PDBu treatment, results in inhibition of AA mitogenic activity. Treatment with this phorbol ester does not affect protein levels of either PKC α, an isoform that requires calcium for its activation [15], or PKC ζ, a DAG and calcium independent isoform [15]. These results suggest that PKC δ and/or PKC ε might have a role in AA stimulation of DNA synthesis in brown adipocytes. Our results also suggest that the α

isoform does not seem to participate in AA stimulation of DNA synthesis. PKC α fail to be completely down regulated in most cell types [15] and, although it has been described an activation of PKC α in vitro by AA [32], it does not translocate to the plasma membrane in response to AA [33]. Thus, our results indicate that PKC is necessary for the AA mitogenic effect and suggest that intracellular signals involving PKC activation could play a central role in the stimulation of brown adipocyte proliferation.

Not only AA but also other polyunsaturated fatty acids of the n-6 family, like the precursor of AA, LA (18:2 n-6), have been found to be mitogenic in other cellular systems [15]. In cultured brown adipocytes we have found that the unsaturated fatty acids LA and OA have low mitogenic activity and the saturated SA or PA did not exert mitogenic effect. Contrary to what could be expected, although LA and LnA has certain mitogenic activity, it is not comparable to the effect exerted by AA. Apart from AA, other unsaturated fatty acids have been shown to activate PKCs [15]. The fact that other unsaturated fatty acids different from AA have no clear mitogenic effect in brown adipocytes confirms the hypothesis that PKCδ could be the target of AA and the molecule responsible for the transmission of the mitogenic signal.

It is known that cAMP-dependent lipolytic stimulation promotes the release of AA from triglycerides in brown adipocytes [19]. It has been shown that white adipose tissue under lipolytic stimulation, releases preferentially short and polyunsaturated fatty acids [34]. Taking into account that brown adipose tissue (BAT) accumulate lipids in small and numerous droplets, the composition of fatty acids in the triglycerides in BAT and WAT must be quite different. In fact, it has been described that BAT is one of the tissues that incorporate more AA from the diet [35]. Thus, it is clear that AA is an important component of brown adipose tissue that can be released in response to the principal regulator of brown adipocyte function, norepinephrine (NE). AA, the main diet n-6 PUFA, is recognized for its role in the stimulation of adipose tissue growth. AA in adipose tissue is also an important constituent of the lipid

droplets and AA content in BAT cells is much higher than in white fat cells. Release of AA may act as a local mediator in the control of brown adipocyte functions suggesting that in addition to its well-known role as an endocrine hormone found in blood serum, AA may also act as a paracrine/ autocrine signaling molecule in BAT. In fact, the AA metabolite 15-deoxy-delta 12,14-prostaglandin J2 is a ligand of the PPARgamma receptor that regulates brown adipocyte differentiation [36]. The importance of AA has been recently reported in lipidomic studies done in obese subjects [37], in which the proportion of AA in the adipose tissue of morbidly obese subjects is markedly decreased.

In conclusion, our results demonstrate the potent mitogenic effect of AA and show the importance of PKC in the stimulation of cellular proliferation in brown adipocytes. Furthermore, the fact that AA behaves as a potent mitogen for brown adipocytes suggest that lipolytic stimulation could initiate signaling pathways directed to stimulate cellular proliferation.

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Legends to figures

Figure 1. Stimulation of ^{3}H-thymidine incorporation by AA in cultured brown preadipocytes. Quiescent brown preadipocytes were treated with 1, 10 or 50 μM AA (panel A), with 10 μM AA, 1 μM NE or both (panel B), with the growth factors EGF (5 ng/ml), PDGF (10 ng/ml), aFGF (1 ng/ml) or bFGF (5 ng/ml) plus 20 nM vasopressin and 1 μM NE, in the absence or presence of 10 μM AA (panel C), with 1, 10 or 50 μM AA, and with 10 μM Indomethacin (Ind) or 4 μM NDGA (panel D), or with 10 and 50 μM of SA, PA, AA, LA, LnA or OA (panel E). Results are expressed as fold increases relative to ^{3}H-thymidine incorporation levels in untreated quiescent cells (dashed line). Data are the means ± SD obtained from two independent experiments using triplicates. For A and B panels: **P*<0.05 *vs* untreated cells. For C panel: **P*<0.05 *vs* no AA. For D panel **P*<0.05 *vs* untreated cells.

Figure 2. **PKC is involved in the stimulation of DNA synthesis by AA and some growth factors.** Quiescent brown preadipocytes were pretreated with 1 μM BIS for 1 hour prior to stimulation with 10 μM AA (Panel A); 40 nM PDBu or 200 nM TPA (Panel B); 5 ng/ml EGF, 20 nM vasopressin (Vas), 1 ng/ml aFGF, 5 ng/ml bFGF, or 1μM NE (Panel C). Results are expressed as fold increases relative to ^{3}H-thymidine incorporation levels in untreated quiescent cells (dashed line). Data are the means ± SD obtained from 2 independent experiments performed in triplicates. For A, B and C panels: **P*<0.05 *vs* untreated cells.

Figure 3. **Down-regulation of PKCδ and ε inhibits the stimulation of DNA synthesis by AA.** Panel A: Quiescent brown preadipocytes were pretreated with 0.2 or 2 μM PDBu for 24 h and cells harvested for protein analysis. A representative Western blot performed with 50 μg of total protein extracts is shown. Panel B and C: Quiescent brown preadipocytes were pretreated with 0.2 μM PDBu for 24 h prior to the addition of PDBu at different concentrations (Panel B: 40 and 400 nM in PDBu pretreated cells; 20, 40, 100, 200 and 400 nM in non-treated cells), or 10 μM AA (Panel C). Results are expressed as fold increases relative to ^{3}H-thymidine incorporation levels in untreated quiescent cells (dashed line). Data are the means ± SD from

two experiments performed in triplicates. *P<0.05 *vs* cells non-pretreated with PDBu.

Figure 4. **MEK/ERK pathway is involved in the mitogenic action of AA.** Panel A: Quiescent brown preadipocytes were depleted for insulin the last 24 h and then treated with the MEK inhibitor PD98059 (50 μM) for 1 h, prior to addition of 10 μM AA, 3 μM insulin (Ins) or 50 nM PDBu for 15 min. Total protein extracts (30 μg) were used for Western blot determination of phospho ERK1/2 levels. ERK1/2 inmunodetection is shown as loading control. A representative Western blot is shown. Panel B and C: Quiescent brown preadipocytes were treated with PD98059 (50 μM) for 1 h and then stimulated with AA (10 μM). After 40 h cells were harvested for ^{3}H-thymidine incorporation measurements (Panel B) or counted by the trypan blue exclusion method. Results are expressed as fold increases relative to ^{3}H-thymidine incorporation levels in untreated quiescent cells (Panel B) or as total cell number (Panel C). Data are the means±SD obtained from two independent experiments performed in triplicates. *P<0.05 *vs* cells non-treated with PD98059.

Figure 5. **PKCδ is required for AA-dependent stimulation of DNA synthesis and ERK1/2 phosphorylation.** Panel A and B: Quiescent brown preadipocytes were depleted for insulin the last 24 h and then treated for 1 h with the specific inhibitors for PKCε, PKCδ and PKCζ at 5 μM (panel A) or 0.5 μM rottlerin (PKCδ inhibitor) (panel B), prior to addition of 50 nM PDBu or 10 μM AA for 15 min. Total protein extracts (30 μg) were used for Western blot determination of phospho ERK1/2 levels. ERK1/2 inmunodetection is shown as loading control. A representative Western blot is shown. Panel C and D: Quiescent brown preadipocytes were treated with the PKCδ specific inhibitor (rottlerin) for 1 h at different concentrations (0.5, 1 or 5 μM; panel C), or with the specific inhibitor for PKCε added at 1 or 10 μM (panel D). Cell cultures were then stimulated with AA (10 μM) or PDBu (50 nM). After 40 h cells were harvested for ^{3}H-thymidine incorporation measurements. Results are expressed as fold increases relative to ^{3}H-thymidine incorporation levels in untreated quiescent

cells. Data are the means ± SD obtained from two independent experiments performed in triplicates.

Figure 1new

Figure 1

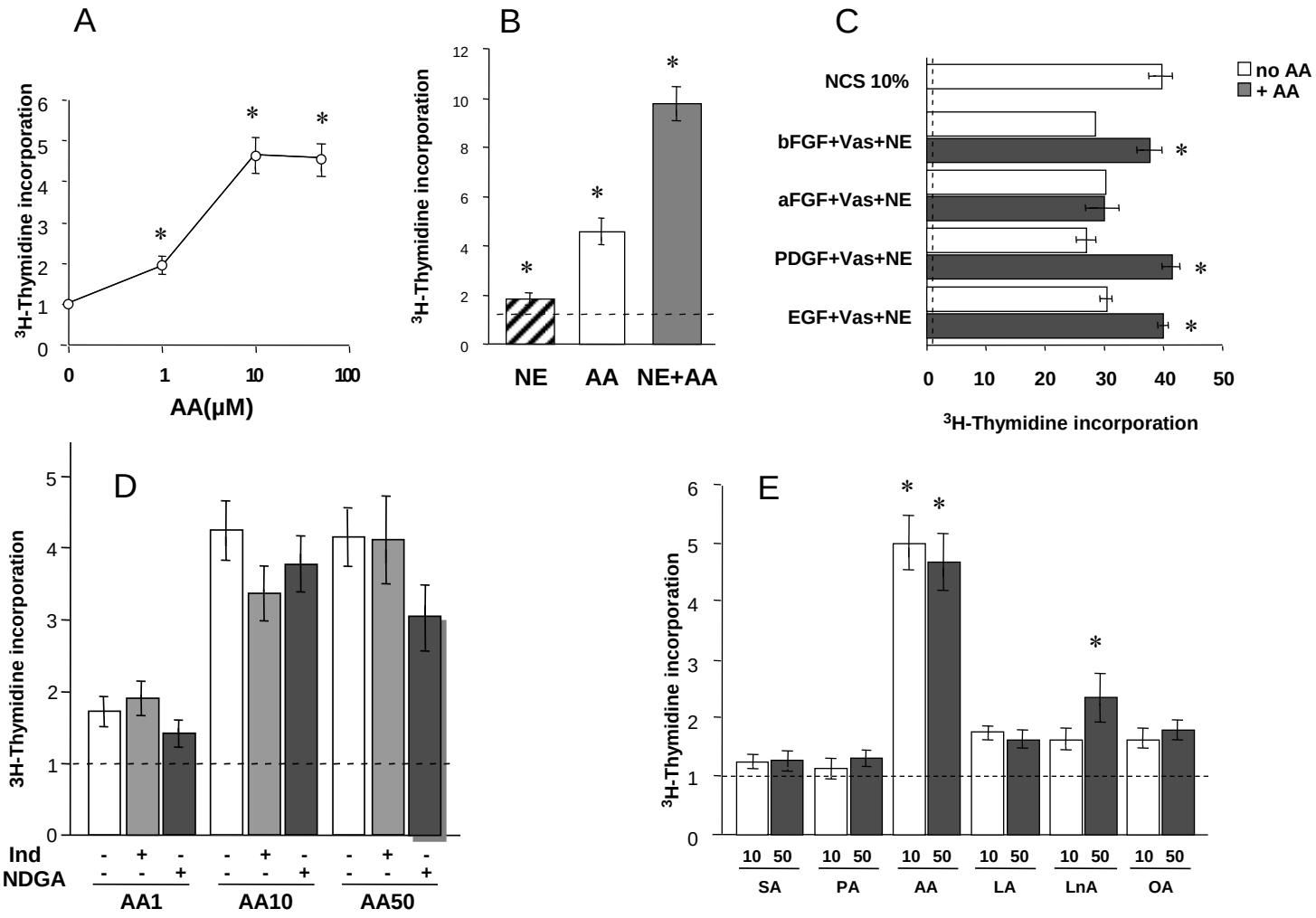

Figure 2

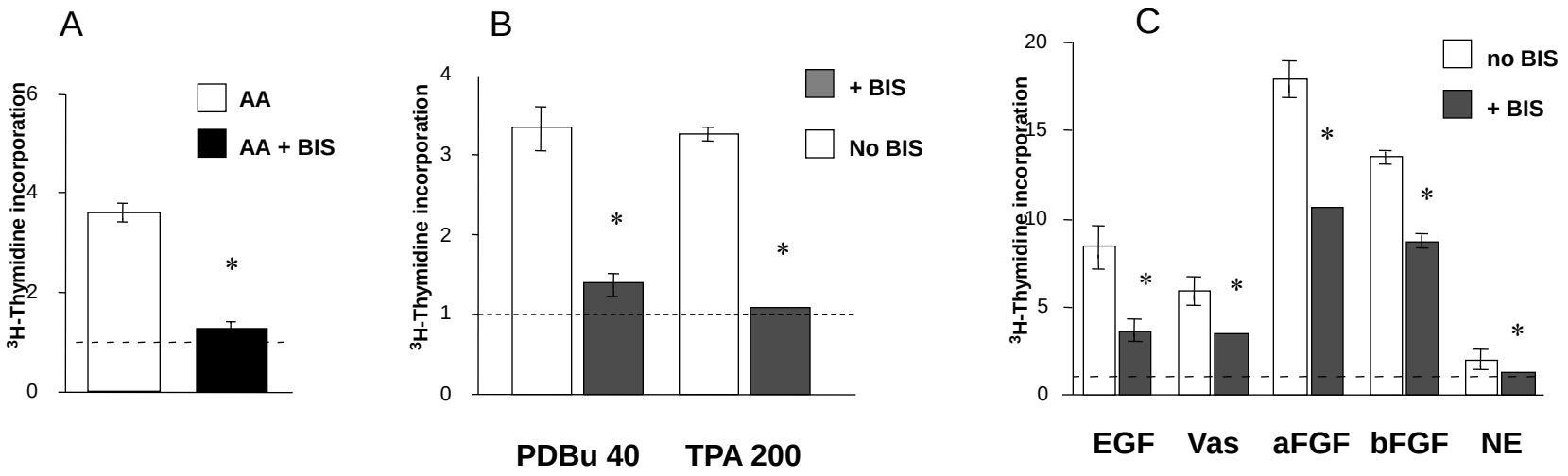
A
B
C
^{3}H-Thymidine incorporation
AA
AA + BIS
+ BIS
No BIS
no BIS
PDBu 40
TPA 200
EGF
Vas
aFGF
bFGF
NE

Figure 3

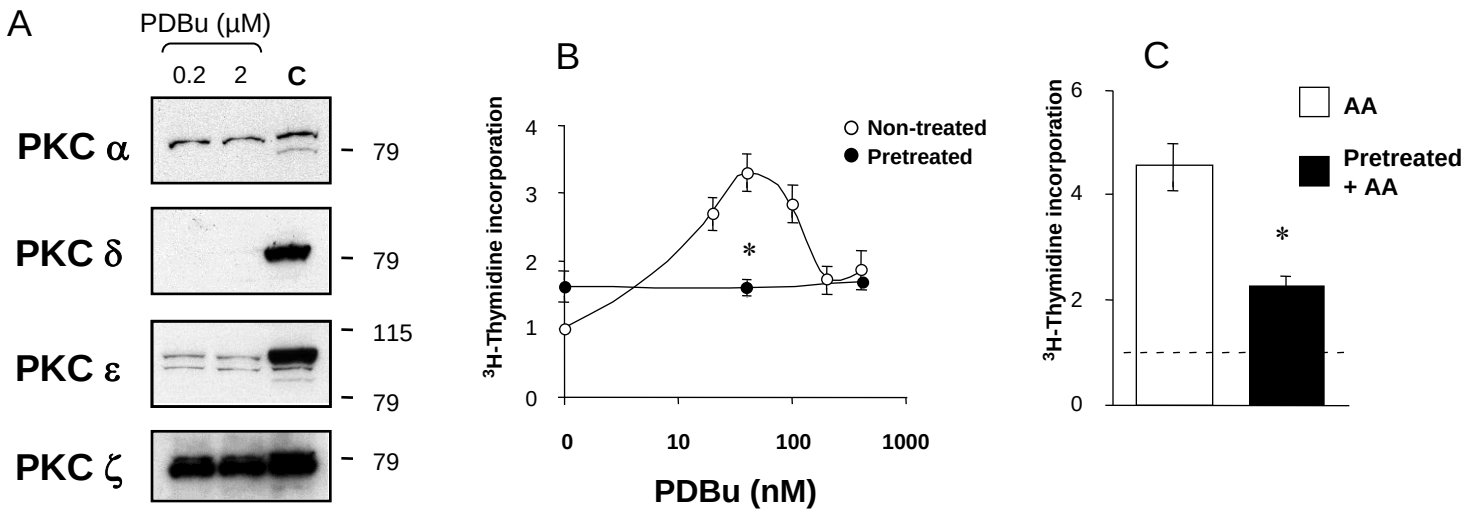
A
PDBu (μM)
0.2
2
C
PKC α
PKC δ
PKC ε
PKC ζ
79
79
115
79
79
B
Non-treated
Pretreated
³H-Thymidine incorporation
PDBu (nM)
0
10
100
1000
C
AA
Pretreated + AA
³H-Thymidine incorporation

Figure 4new

Figure 4

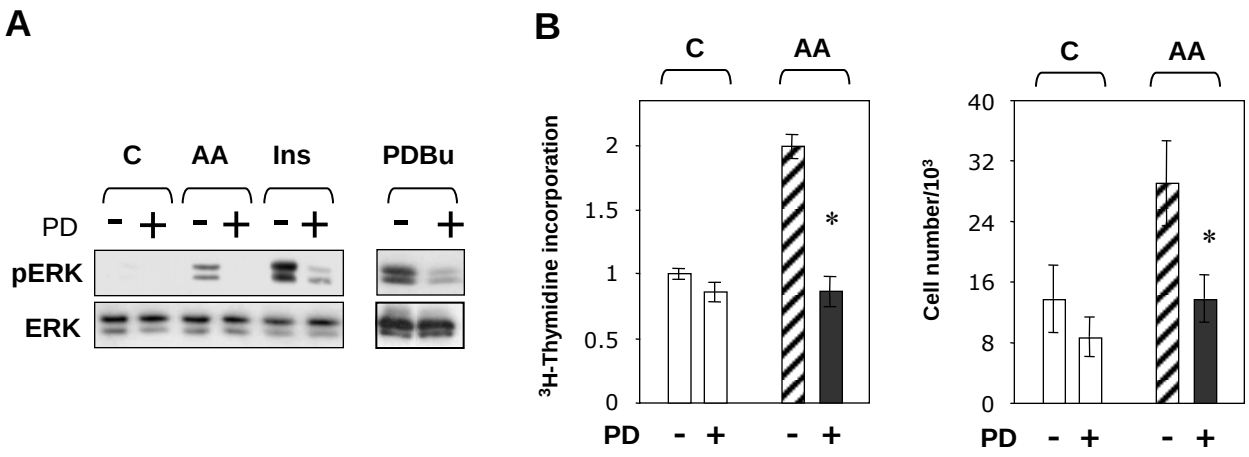

Figure 5new

Figure 5